

## RESEARCH ARTICLE

# Knockdown of kinesin family member 4A inhibits cell proliferation, migration, and invasion while promoting apoptosis of urothelial bladder carcinoma cells

Chen Zhang<sup>1</sup>  | Maoyu Wang<sup>1</sup> | Yidie Ying<sup>1</sup> | Fang Meng<sup>1</sup> | Hongliang Gao<sup>1</sup> | Shuxiong Zeng<sup>1</sup> | Yasheng Zhu<sup>1</sup> | Anwei Liu<sup>2</sup> | Zhensheng Zhang<sup>1</sup>  | Chuanliang Xu<sup>1</sup>

<sup>1</sup>Department of Urology, Changhai Hospital, Naval Military Medical University, Shanghai, 200433, China

<sup>2</sup>Department of Critical Care Medicine, Hospital of Southern Theatre Command of PLA, Guangzhou, 510010, China

## Correspondence

Zhensheng Zhang and Chuanliang Xu, Department of Urology, Changhai Hospital, Naval Military Medical University, Changhai Road, Yangpu District, Shanghai 200433, China.  
Email: [zhangzs137@163.com](mailto:zhangzs137@163.com) and [chuanliang\\_xu@126.com](mailto:chuanliang_xu@126.com)

## Funding information

the "Voyaging Talents" Fund of The PLA Naval Medical University, Grant/Award Number: 2021008149; National Natural Science Foundation of China, Grant/Award Number: 81772720 and 81972391

## Abstract

**Background:** Kinesin family member 4A (KIF4A) is upregulated in a variety of cancers. However, its expression and potential downstream targets in urothelial bladder carcinoma (UBC) remain unclear.

**Methods:** Expression data of KIF4A in UBC and noncancerous tissues were downloaded from the GEPIA database. Cell proliferation, migration, invasion, and apoptosis of T24 and 5637 UBC cells were examined using wound healing, transwell, colony formation, CCK-8, and flow cytometry assays. KIF4A and potential downstream genes were analyzed using qRT-PCR, western blot analysis, and immunohistochemistry.

**Results:** In UBC samples, KIF4A expression was significantly higher than in corresponding noncancerous samples. UBC patients with high KIF4A expression had poor cancer-specific survival and overall survival. Knockdown of KIF4A significantly inhibited proliferation and promoted apoptosis of UBC cells, accompanied by dephosphorylation of AKT and increased the protein level of proapoptotic factors. Additionally, knockdown of KIF4A reduced migration and invasion of UBC cells whereas overexpression of KIF4A exhibited opposite effects, along with altered protein level in epithelial-mesenchymal transition-related genes. Furthermore, overexpression of YAP1 promoted KIF4A expression whereas knockdown of YAP1 suppressed KIF4A expression in UBC cells. Alternatively, KIF4A knockdown reduced YAP1 nuclear protein level whereas KIF4A overexpression suppressed YAP1 phosphorylation and facilitated YAP1 nuclear translocation.

**Conclusions:** KIF4A upregulation correlates with poor prognosis of UBC. Knockdown of KIF4A inhibits proliferation, migration, and invasion of UBC cells

Chen Zhang, Maoyu Wang, and Yidie Ying contributed equally to this work.

This is an open access article under the terms of the [Creative Commons Attribution](https://creativecommons.org/licenses/by/4.0/) License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2023 The Authors. *Cancer Medicine* published by John Wiley & Sons Ltd.

while inducing apoptosis possibly through dephosphorylation of AKT, changes in EMT-related genes, and interaction with YAP1.

#### KEYWORDS

AKT serine/threonine kinase, kinesin family member 4A, urothelial bladder carcinoma, Yes1 associated transcriptional regulator

## 1 | INTRODUCTION

Urothelial bladder carcinoma (UBC) is the most common neoplasm of the urinary system.<sup>1</sup> Approximately 5%–10% of UBC cases present metastases at the time of diagnosis. After radical cystectomy, about 50% of patients experience local or distant tumor relapse.<sup>2</sup> Despite the development of therapeutic approaches, nearly 50%–70% of cases relapse within 5 years of recovery.<sup>3</sup> Therefore, it is important to identify new therapeutic targets for UBC.

Kinesin family member 4A (KIF4A) is located on chromosome Xq13.1, encoding a protein containing 1232 amino acids. KIF4A participates in multiple cellular processes, such as centrosome assembly and spindle formation in mitosis, chromosomal separation and concentration, as well as DNA damage repair.<sup>4</sup> KIF4A dysregulation occurs in various tumors, including lung,<sup>5</sup> liver,<sup>6</sup> breast,<sup>7</sup> and bladder cancer.<sup>8</sup> Yes1 associated transcriptional regulator (YAP1) is an important component of the Hippo signaling pathway that is highly associated with cell growth. YAP1 induces KIF4A transcription by binding to the KIF4A promoter via TEA domain transcription factor 4 (TEAD4), contributing to the progression and poor prognosis of esophageal squamous cell carcinoma.<sup>9</sup> However, the role of KIF4A in UBC metastasis and the underlying mechanism of KIF4A dysregulation in UBC remain largely unknown.

Abnormal or incomplete mitosis may induce cell apoptosis. KIF4A silencing may lead to apoptosis by inducing spindle defects, dysfunction in prometaphase organization, metaphase chromosomal misalignment, and chromosomal mis-segregation.<sup>10</sup> A recent study has shown that KIF4A silencing reduces p-AKT level in gastric cancer.<sup>11</sup> Since AKT regulates cell proliferation and apoptosis by phosphorylating multiple downstream targets, such as P21, Bax, and Bcl-2,<sup>12</sup> we hypothesized that KIF4A might affect the apoptosis of UBC cells through AKT.

In this study, we investigated the expression of KIF4A in human UBC and the role of KIF4A in UBC cell proliferation, migration, invasion, and apoptosis. To identify the potential mechanisms underlying the role of KIF4A in UBC development, we examined the alterations in the protein level of apoptosis and epithelial-mesenchymal

transition (EMT)-related genes as well as the interaction between KIF4A and YAP1. Our results suggest that KIF4A contributes to UBC development and metastasis possibly through the activation of AKT and interaction with YAP1.

## 2 | MATERIALS AND METHODS

### 2.1 | Specimens and data collection

KIF4A expression data of 404 UBC tumor samples and 28 noncancerous tissue samples were acquired from the (GEPiA) Gene Expression Profiling Interactive Analysis database. A total of 26 UBC patients who underwent radical cystectomy between June 2013 and May 2021 at the department of urology at The First Affiliated Hospital of Naval Medical University (Shanghai, China) were recruited to validate differential expression of KIF4A between bladder cancer and noncancerous tissue samples. RNA sequencing was used to validate the differential expression of KIF4A in 10 patients admitted between June 2013 and May 2014 (Table S1), and western blot analysis was used to validate the differential expression in 16 patients admitted between May 2020 and May 2021 (Table S2). A total of 232 bladder cancer and adjacent noncancerous specimens were obtained from our hospital for immunohistochemistry (IHC) analysis (Table 1). A tissue microarray was prepared. The specimens were collected during transurethral resection of bladder tumors or radical cystectomy from 1998 to 2018, including 148 muscle-invasive bladder cancer, 84 non-muscle-invasive bladder cancer, 185 high-grade bladder cancer, and 47 low-grade bladder cancer.

### 2.2 | Cell lines and cell culture

UBC cell lines were obtained from American Type Culture Collection (Manassas) and cultured in DMEM (T24 and HT-1376 cells), RPMI-1640 (5637 cells), MEM (J82 and UM-UC-3 cells), or L15 medium (BIU-87 cells) supplemented with 10% of fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific) in an atmosphere of 5% CO<sub>2</sub> at 37°C.

**TABLE 1** Association of KIF4A expression with clinicopathological characteristics of patients with urothelial bladder carcinoma.

Variables	KIF4A expression		p-value ( $\chi^2$ test)
	Low (n = 126)	High (n = 106)	
Gender			
Male	113 (54.07%)	96 (45.93%)	0.822
Female	13 (56.52%)	10 (43.48%)	
Age, years			
≤65	27 (57.45%)	20 (42.55%)	0.743
>65	99 (53.51%)	86 (46.49%)	
Tumor size			
<3 cm	48 (64.00%)	27 (36.00%)	0.048
≥3 cm	78 (49.68%)	79 (50.32%)	
Tumor stage			
<T2	61 (72.62%)	23 (27.38%)	<0.001
T2–T4	65 (43.92%)	83 (56.08%)	
Tumor grade			
Low	32 (68.09%)	15 (31.91%)	0.048
High	94 (50.81%)	91 (49.19%)	
Recurrence history			
No	92 (57.86%)	67 (42.14%)	0.120
Yes	34 (46.58%)	39 (53.42%)	

### 2.3 | Preparation of nuclear and cytoplasmic extract

Nuclear and cytoplasmic extracts were prepared using NEPER™ nuclear and cytoplasmic extraction kit (Thermo Scientific). Pre-chilled 200  $\mu$ L CER1 reagent was added to the cells, followed by vortex shaking at high speed for 15s. After incubation on ice for 10min, 11  $\mu$ L of CERII reagent was added to the cells, vortexed for 5s, then incubated on ice for 1min. After centrifuging at 16,000rpm for 5min, the supernatant was transferred to a fresh pre-cooled centrifuge tube to store on ice or at  $-80^{\circ}\text{C}$  for further experiments. 100  $\mu$ L of pre-chilled NER was added to the precipitate, resuspended with high speed vortex for 15s, incubated on ice for 10min, and vortexed again at high speed for 15s. This process was repeated four times. The nucleoplasmic protein extract was obtained by centrifuging the solution at 16,000rpm for 10min and keep on ice or stored at  $-80^{\circ}\text{C}$  for subsequent experiments.

### 2.4 | Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was isolated from UBC cells using TRIzol reagent (Invitrogen), followed by cDNA synthesis using an

all-in-one RT super-mix perfect kit (Vazyme) according to the manufacturer's protocol. PCR was performed using a Taq Pro universal SYBR qPCR master mix (Vazyme) on Applied Biosystems step one plus (Agilent Technologies). Relative gene expression was determined using the  $2^{-\Delta\Delta\text{CT}}$  method. The primer sequences were as follows: KIF4A 5'-TGGTGTGGAAACAAGCAGTGTG-3' (forward), 5'-GGAATCCTGGGTCCGTTCA-3' (reverse); ITGB1, 5'-CCTACTTCTGCACGATGTGATG-3' (forward), 5'-CCTTTGCTACGGTTGGTTACATT-3' (reverse); GAPDH, 5'-ACCACAGTCCATGCCATCAC-3' (forward), 5'-TCCACCACCCTGTTGCTGTA-3' (reverse);  $\beta$ -actin, 5'-CATGTACGTTGCTATCCAGGC-3' (forward), 5'-CTCCTTAATGTCACGCACGAT-3' (reverse).

### 2.5 | IHC

IHC was performed to detect KIF4A expression in the tissue microarray containing 232 UBC tumor tissue samples. The microarray was incubated with anti-KIF4A primary antibody (1:400; ab122227, Abcam) and stained using an IHC kit (BioGenex) following the manufacturer's protocol. The IHC scores of the cytoplasm and nuclei were calculated, respectively, as 0  $\times$  % non-stained + 1  $\times$  % weakly stained + 2  $\times$  % moderately stained + 3  $\times$  % strongly stained, ranging from 0 to 300.<sup>13</sup> Patients were divided into high and low KIF4A expression groups according to the median (240) of the sum of cytoplasmic IHC score and nuclear IHC score.<sup>13</sup>

### 2.6 | Gene knockdown and overexpression

Small interference RNAs (siRNAs) against KIF4A or YAP1 were purchased from GenePharma. The siRNA sequences were as follows: KIF4A: siRNA-1: sense, 5'-CUGCAGAGCAAGAGAAUGATT-3', antisense, 5'-UCAUUCUCUUGCUCUGCAGUA-3'; siRNA-2: sense, 5'-CAGCAAAGAAGGAUGCCAATT-3', antisense, 5'-UUGGCAUCCUUCUUUGCUGUC-3'; siRNA-3: sense, 5'-GCUGGUUGAGUUGAAUAAATT-3', antisense, 5'-UUUAUUCAACUCAACCAGCUC-3'; nonspecific siRNA: sense, 5'-UUCUCGGAACGUGUCACGUTT-3', antisense, 5'-ACGUGACACGUUCGGAGAATT-3'; YAP1 siRNA: sense, 5'-CAGGU GAUACUAUCAACCATT-3', antisense, 5'-UGGUU GAUAGUAUCACCUGTT-3'. Lentiviral vectors expressing KIF4A (#31640-1) or YAP1 (#14517-2) were purchased from Genechem. UBC cells were transfected with siRNAs or lentiviral vectors using RNAiMAX or lipofectamine (Invitrogen) following the manufacturer's instructions.

## 2.7 | Western blot analysis

Protein concentration was measured using the BCA method. Equal amount of each protein sample was separated on SDS-PAGE and transferred onto PVDF membranes (Bio-Rad). The membranes were washed five times with TBST, 5 min each, followed by blocking with 5% skimmed milk in TBST for 1 h at 37°C. Then, the membranes were incubated with primary antibody against KIF4A (1:2500, ab124903), GAPDH (1:2500, ab9485),  $\beta$ -actin (1:50000, AC026, Abclonal), ITGB1 (1:4000, 12,594-1-AP, Proteintech), YAP1 (1:5000, ab52771), p-YAP (1:2000, S127, Cell Signaling Technology (Cell Signaling Technology)), AKT (1:2000, 2920, Cell Signaling Technology), p-AKT (Ser473 1:2000, 4060, Cell Signaling Technology), cleaved-caspase 3 (1:2000, 19,677-I-AP, Proteintech), Bax (1:5000, 50,599-2-Ig, Proteintech), Bcl-2 (1:5000, 60,178-1-Ig, Proteintech), PARP (1:2000, 46D11, Cell Signaling Technology), cleaved-PARP (1:1000, Asp214, Cell Signaling Technology), P21 (1:1000, GB11153, Servicebio), E-cadherin (1:1000, ab76055), N-cadherin (1:2000, GB12135, Servicebio), or vimentin (1:1000, GB11192, Servicebio) overnight at 4°C. After TBST washes, the membranes were incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibody (1:3000, Cell Signaling Technology) for 2 h at 4°C. The protein bands were visualized using an enhanced chemiluminescence detection kit (Millipore) and analyzed using the ChemiDoc MP system (Bio-Rad). The relative levels of the proteins were quantified by normalizing the densitometry to controls using ImageJ 1.53.

## 2.8 | Cell counting kit-8 (CCK-8) assay

Cell proliferation was assessed using CCK-8 (Dojindo) following the manufacturer's instructions. Cells were seeded in 96-well plates at a density of  $3 \times 10^3$  cells/well. After treatment, cells were incubated with 20  $\mu$ L CCK-8 reagent for 3 h. The absorbance was measured at a wavelength of 495 nm using an EnSpire microplate reader (PerkinElmer).

## 2.9 | Wound healing assay

UBC cells were seeded in 6-well plates. When the cells achieved 80% confluency, a scratch was made using a 200- $\mu$ L pipette tip. Cells were then cultured in FBS-free medium. Images were acquired at 0, 24, 48, or 72 h after scratching.

## 2.10 | Transwell assay

Cell invasion was assessed using the Matrigel-coated transwell chamber (Millipore). Briefly,  $4 \times 10^4$  cells were seeded in the

upper chamber containing 500  $\mu$ L FBS-free medium. A total of 1 mL of complete medium was added into the lower chamber.

Cell migration was assessed using the transwell chamber (Corning).  $3 \times 10^4$  cells were seeded in the upper chamber containing 500  $\mu$ L FBS-free medium. A total of 1 mL of complete medium was added into the lower chamber. T24 and 5637 cells were allowed to invade and migrate for 18 and 48 h, respectively. Then, the cells remaining in the upper chamber were removed using a cotton swab. Cells adhering to the lower surface were fixed with 4% formalin for 20 min and then stained with 0.1% crystal violet for 30 min. Images were acquired in 10 randomly selected fields, and cells were counted.

## 2.11 | Colony formation assay

Cells were seeded in 3.5-cm plates at a density of  $5 \times 10^2$  cells/well. At 2 weeks after transfection, cells were fixed using 10% methanol, followed by staining with 0.1% crystal violet. Colony formation was analyzed using a microscope.

## 2.12 | Flow cytometry assay

Cells were harvested at 72 h after KIF4A or control siRNA transfection. Cell apoptosis was examined using an Annexin V/FITC and PI apoptosis kit (Lianke Biotech). The results were analyzed using a MACSQuant analyzer and FlowJo v10.8 software.

## 2.13 | Statistical analysis

Data were expressed as the mean  $\pm$  standard error of the mean. Statistical analysis was carried out using Prism software (version 8.4.3). Comparisons of measurement data between two groups were conducted using Student's *t*-test. The chi-squared test was performed to analyze quantitative data and the association of clinicopathological parameters with KIF4A expression level. Overall survival (OS) and cancer-specific survival (CSS) were analyzed using Kaplan–Meier analysis and log-rank test. A *p* value  $< 0.05$  (two-sided) was considered statistically significant.

## 3 | RESULTS

### 3.1 | KIF4A is upregulated in UBC and associated with poor prognosis of UBC patients

To explore the involvement of KIF4A in UBC, we compared KIF4A mRNA expression between bladder cancer



tissue and normal bladder tissue from the GEPIA. We found that KIF4A mRNA expression was remarkably up-regulated in bladder cancer tissue compared with that in normal bladder tissue (Figure 1A). This was confirmed in the samples of 10 UBC patients from our hospital (Figure 1B,C). Consistently, in 16 UBC patients at our hospital, KIF4A protein was generally highly expressed in tumor tissue but barely detectable in paired normal bladder tissue (Figure 1D,E). In addition, KIF4A protein was extensively expressed in UBC cell lines, including BIU87, 5637, J82, UMUC3, HT1376, and T24, as well as in normal urothelial cells SVHUC. Specifically, KIF4A protein level in BIU87, T24, J82, and UMUC3 cells were markedly increased compared with that in SVHUC cells ( $p < 0.05$ ; Figure 1F,G). Furthermore, IHC staining showed that KIF4A protein was expressed in both nuclear and cytoplasm of UBC cells (Figure 1H). Based on the mean IHC score, UBC patients were categorized into low (IHC score  $\leq 240$ ,  $n = 126$ ) and high (IHC score  $> 240$ ,  $n = 106$ ) KIF4A expression groups. We found that high KIF4A expression was significantly associated with tumor size  $> 3$  cm ( $p = 0.048$ ) and advanced tumor grade ( $p = 0.048$ ) and tumor stage ( $p < 0.001$ ) (Table S1). Survival analysis showed that high KIF4A expression was significantly correlated to poor CSS ( $p = 0.016$ ) and OS ( $p = 0.009$ ) (Figure 1I). These results suggest that KIF4A upregulation is associated with disease severity and poor prognosis of patients with UBC.

### 3.2 | Knockdown of KIF4A inhibits proliferation while promoting apoptosis of UBC cells

To explore the role of KIF4A in UBC, we silenced KIF4A expression in 5637 (Figure 2A) and T24 (Figure 2B) cells, respectively, and found that siRNA-2 exhibited the most significant knockdown efficiency among three siRNAs. Colony formation assay and CCK-8 assay showed that knockdown of KIF4A considerably inhibited cell proliferation of both cell lines (Figure 2C-F). Meanwhile, knockdown of KIF4A markedly enhanced cell apoptosis of both UBC cell lines (Figure 2G,H). Of note, siRNA-2 outperformed siRNA-1 in promoting apoptosis of UBC cells. These data suggest that KIF4A is required for the survival of UBC cells.

### 3.3 | Knockdown of KIF4A deactivates AKT signaling in UBC cells

Because KIF4A silencing reduces p-AKT level in gastric cancer,<sup>11</sup> we investigated the effect of KIF4A silencing

on AKT activation and the protein level of downstream apoptosis-related genes in UBC cells. Western blot analysis showed that knockdown of KIF4A substantially attenuated AKT phosphorylation in both UBC cell lines, with the total AKT protein level remaining unchanged. In the meantime, knockdown of KIF4A significantly enhanced the protein level of proapoptotic cleaved caspase 3, Bax, cleaved PARP, and p21 while suppressing protein level of cell proliferation-related Bcl-2 and p-YAP1 in both cell lines (Figure 2I,J). Again, siRNA-2 was superior to siRNA-1 in AKT dephosphorylation.

### 3.4 | KIF4A promotes cell migration and invasion of UBC cells

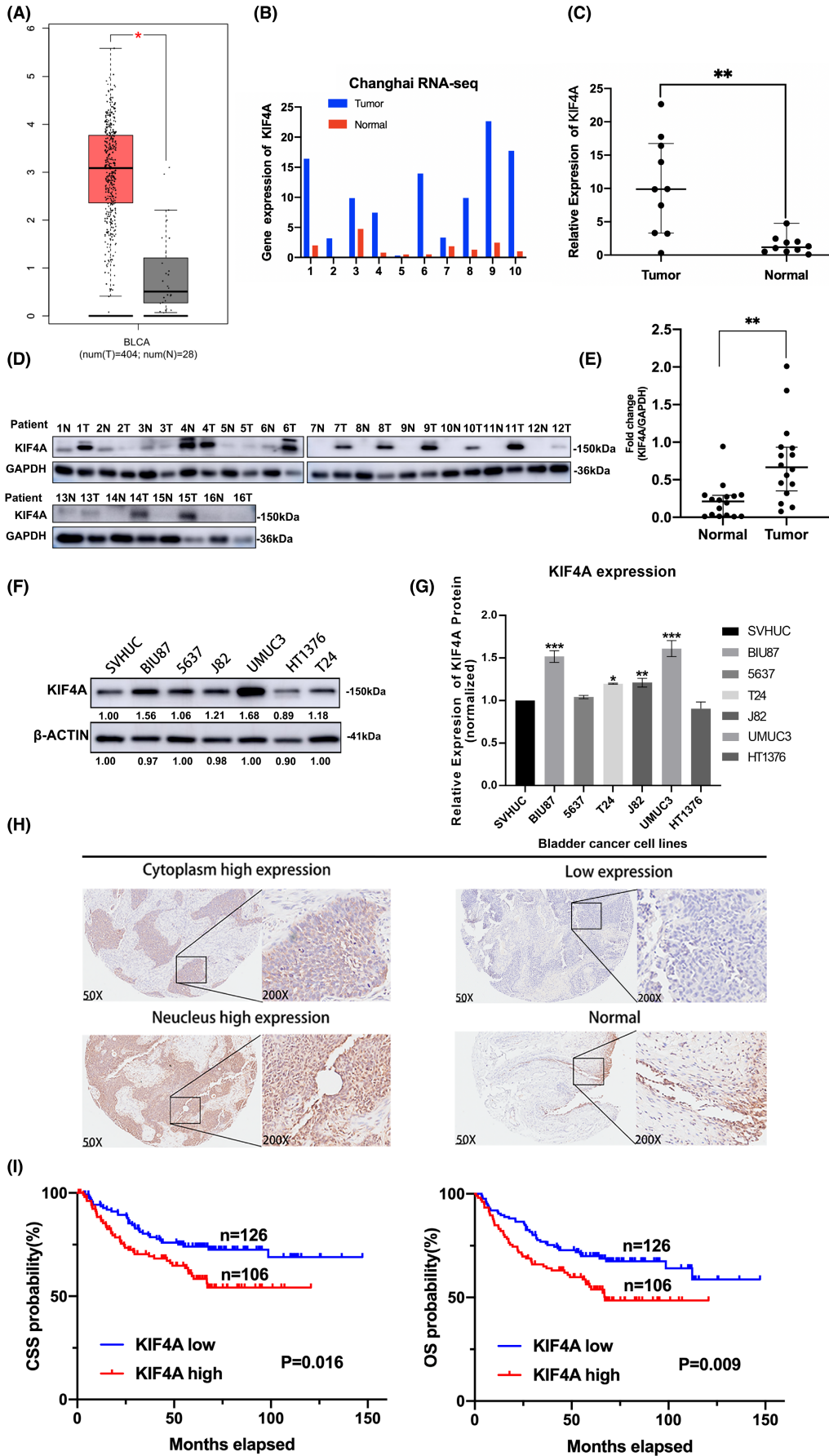
Then, we explored the role of KIF4A in UBC cell migration and invasion. Transwell and wound healing assays showed that KIF4A silencing markedly reduced cell invasion and migration of both T24 and 5637 cells (Figure 3A-D). On the contrary, KIF4A overexpression (Figure 3E) substantially promoted cell invasion and migration of 5637 cells (Figure 3E-H). Time-dependent trends were observed in the promoting effect of KIF4A overexpression on migration of 5637 cells (Figure 3H) and the inhibitory effect of KIF4A silencing on migration of both T24 and 5637 cells (Figure 3I,J).

### 3.5 | Knockdown of KIF4A modulates EMT-related genes in UBC cells

Increased ability of migration and invasion are linked to EMT that is a key step in cancer metastasis.<sup>14</sup> Thus, we sought to investigate whether KIF4A regulates the protein level of EMT-related genes. We found that knockdown of KIF4A significantly suppressed protein level of N-cadherin, vimentin, and integrin subunit beta 1 (ITGB1) while enhancing protein level of E-cadherin in UBC cells (Figure 4A,B). By contrast, overexpression of KIF4A significantly boosted protein level of N-cadherin, vimentin, and ITGB1 while diminishing protein level of E-cadherin in UBC cells. In the meantime, overexpression of KIF4A dramatically increased AKT phosphorylation while reducing YAP1 phosphorylation (Figure 4C). These data suggest that KIF4A can potentially facilitate UBC metastasis by promoting EMT of UBC cells.

### 3.6 | KIF4A interacts with YAP1 in UBC

Considering the regulatory role of YAP1/TEAD4 in KIF4A transcription in esophageal squamous cell carcinoma and



**FIGURE 1** Kinesin family member 4A (KIF4A) was upregulated in urothelial bladder carcinoma (UBC) and associated with poor prognosis of UBC patients. (A) Comparison of KIF4A mRNA expression between bladder cancer samples ( $n=404$ ) and normal urothelium samples ( $n=28$ ) from the GEPIA (Gene Expression Profiling Interactive Analysis database). T, tumor; N, normal. (B,C) Comparison of KIF4A mRNA expression between bladder cancer samples and normal urothelial samples from 10 UBC patients by RNA sequencing. (D,E) Western blot analysis was conducted to determine KIF4A protein expression in 16 pairs of bladder cancer samples and noncancerous samples. T, tumor; N, noncancerous. GAPDH was used as an internal reference. (F,G) Western blot analysis was conducted to measure KIF4A protein expression in normal urothelial cell line SVHUC, non-muscle-invasive bladder cancer cell line BIU-87, and five different muscle-invasive bladder cancer cell lines (5637, J82, UMUC3, HT1376, and T24).  $\beta$ -Actin was used as an internal reference. Data are expressed as the mean  $\pm$  standard error of the mean (SEM). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ;  $n = 3$ . (H) Immunohistochemical staining was performed to determine KIF4A expression in human UBC samples and normal urothelial samples. Representative images are shown. UBC patients were categorized into low (IHC score  $\leq 240$ ,  $n = 126$ ) and high (IHC score  $> 240$ ,  $n = 106$ ) KIF4A expression groups. (I) Survival analysis was conducted to examine the correlation of KIF4A expression with cancer-specific survival and overall survival of patients. CSS, cancer-specific survival; OS, overall survival.

the role of TEAD1 in chemoresistance of bladder cancer,<sup>9,15</sup> we sought to investigate whether YAP1/TEAD1 is involved in KIF4A dysregulation in UBC. We found that overexpression of YAP1 resulted in a 2.14-fold increase in KIF4A protein level whereas knockdown of YAP1 or TEAD1 led to a  $< 50\%$  reduction in KIF4A protein level in UBC cells (Figure 4D). These data suggest that YAP1/TEAD1 are at least partially responsible for KIF4A upregulation in UBC. Cytoskeleton proteins can bind to phosphorylated YAP1 (p-YAP1), inducing its cytoplasmic translocation and degradation and subsequent inhibition of cell proliferation.<sup>16</sup> Our immunofluorescence data demonstrated that knockdown of KIF4A dramatically reduced YAP1 nuclear protein level in 5637 cells (Figure 4E), suggesting an interaction between KIF4A and YAP1 in UBC. Furthermore, western blot analysis showed that overexpression of KIF4A significantly suppressed YAP1 phosphorylation in both cytoplasm and nucleus of 5637 cells and remarkably increased YAP1 protein level in the nucleus. Opposite effects were observed in KIF4A-silenced cells (Figure 4F). These data suggest that YAP1 is involved in KIF4A upregulation in UBC and that KIF4A contributes to UBC cell proliferation possibly by promoting YAP1 dephosphorylation and nuclear translocation.

#### 4 | DISCUSSION

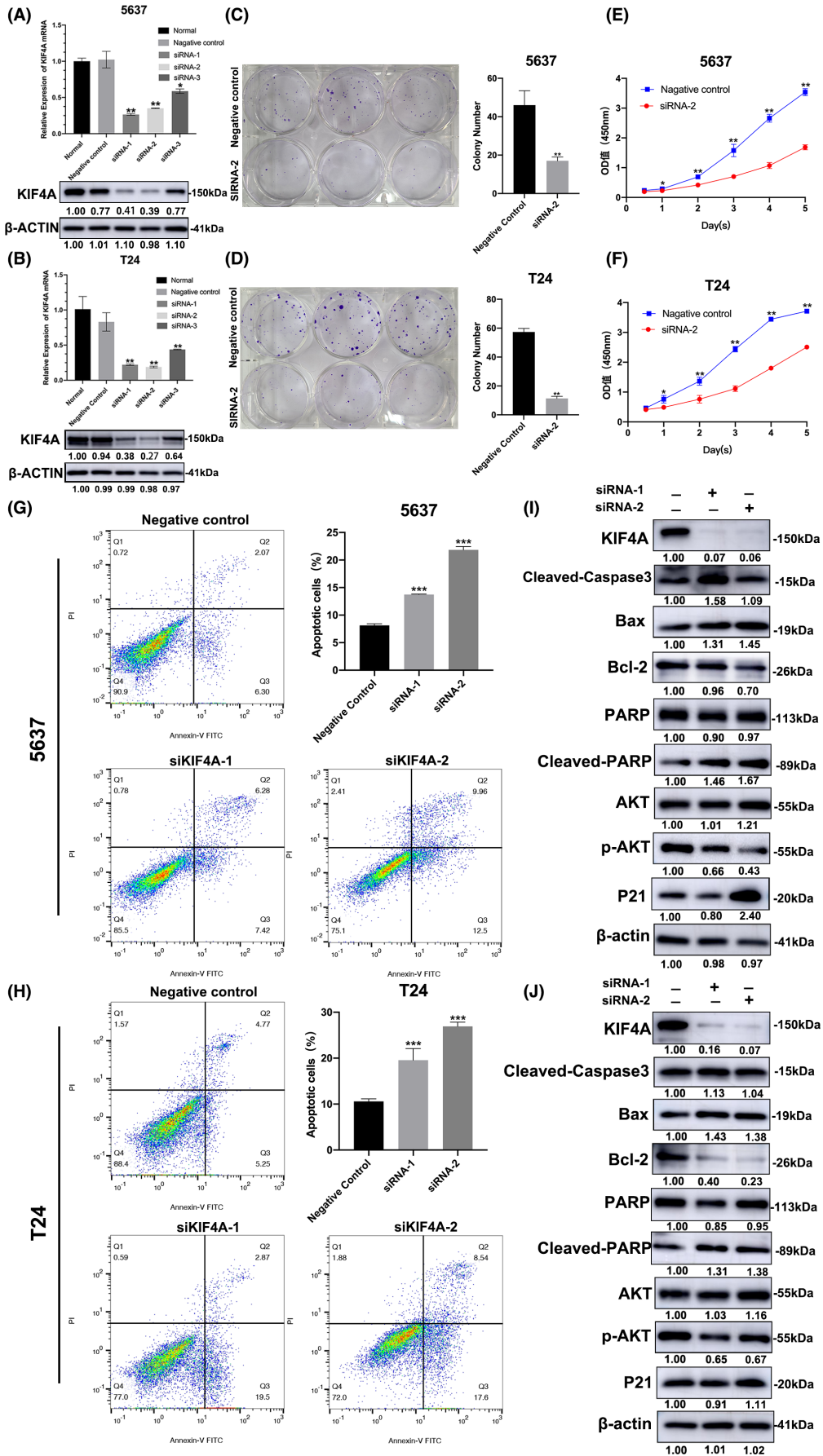
This study explored KIF4A expression in human UBC and the role of KIF4A in UBC cell proliferation, migration, invasion, and apoptosis. We demonstrated that KIF4A protein was strongly expressed in human UBC tumor tissue and UBC cell lines compared with that in noncancerous tissue and normal urothelial cells, respectively. High KIF4A expression was significantly correlated with poor survival of patients with UBC. In vitro studies showed that knockdown of KIF4A hindered cell proliferation while promoting apoptosis of UBC cells. On the contrary, overexpression of KIF4A facilitated UBC cell migration and invasion. At the molecular level, knockdown of KIF4A

reduced AKT phosphorylation and modulated EMT-related proteins in UBC cells. Furthermore, KIF4A interacted with YAP1 in UBC cells. Taken together, our study suggests that KIF4A may promote UBC growth and metastasis, serving as a potential therapeutic target for UBC.

Apoptosis involves two major pathways, namely intrinsic and extrinsic pathway. Caspases-3 is an important proapoptotic molecule and can cleave downstream targets such as PARP and P21.<sup>17</sup> Bcl-2 and Bax are the two factors that mediate the intrinsic apoptotic pathway. Bcl-2 dissociated from Bax triggers the intrinsic apoptosis by modulating mitochondrial function.<sup>18</sup> In this study, KIF4A silencing suppressed Bcl-2 protein level while enhancing Bax protein level in UBC cells, accompanied with elevations in cleaved caspase-3, cleaved PARP, and P21 protein level. Similarly, Zhang et al. have reported that knockdown of KIF4A inhibits tumor progression and promotes chemosensitivity of lung cancer cells by inducing P21 expression.<sup>19</sup> Jin et al. have observed diminished Bcl-2 protein level and increased Bax and cleaved caspase3 protein level in KIF4A-silenced apoptotic ovarian cancer cells.<sup>20</sup> Thus, targeting KIF4A is a promising therapeutic approach to cancer therapy by inducing apoptosis of tumor cells.

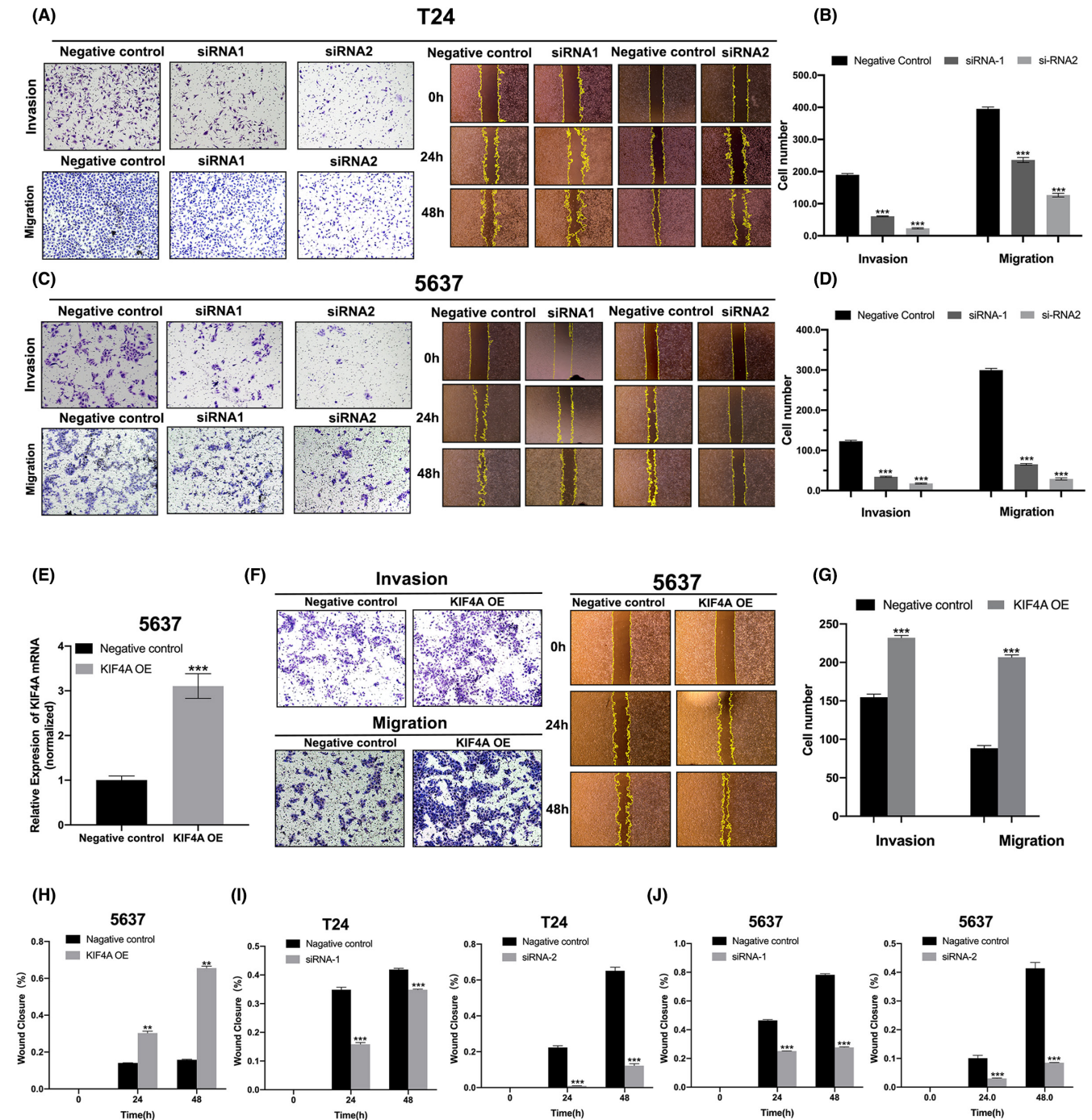
The transcription coactivator YAP1 is an important molecule in the Hippo pathway that regulates cell proliferation. In esophageal squamous cell carcinoma, KIF4A inhibits YAP1 phosphorylation, leading to enhanced nuclear YAP1 protein level.<sup>21</sup> Consistently, we found that overexpression of KIF4A suppressed YAP1 phosphorylation and promoted YAP1 nuclear translocation in 5637 cells. In addition, YAP1 can modulate the transcription of anti-apoptotic genes, such as the Bcl-2 family member Bcl-xL.<sup>22</sup> We speculated that the Bcl-2 downregulation in response to KIF4A silencing might be related to the reduction in YAP1 protein level. These observations suggest that KIF4A silencing promotes UBC cell apoptosis possibly by regulating the phosphorylation and cytoplasmic/nuclear translocation of YAP1.

AKT activation suppresses cytochrome c production through direct phosphorylation of Bax and subsequent

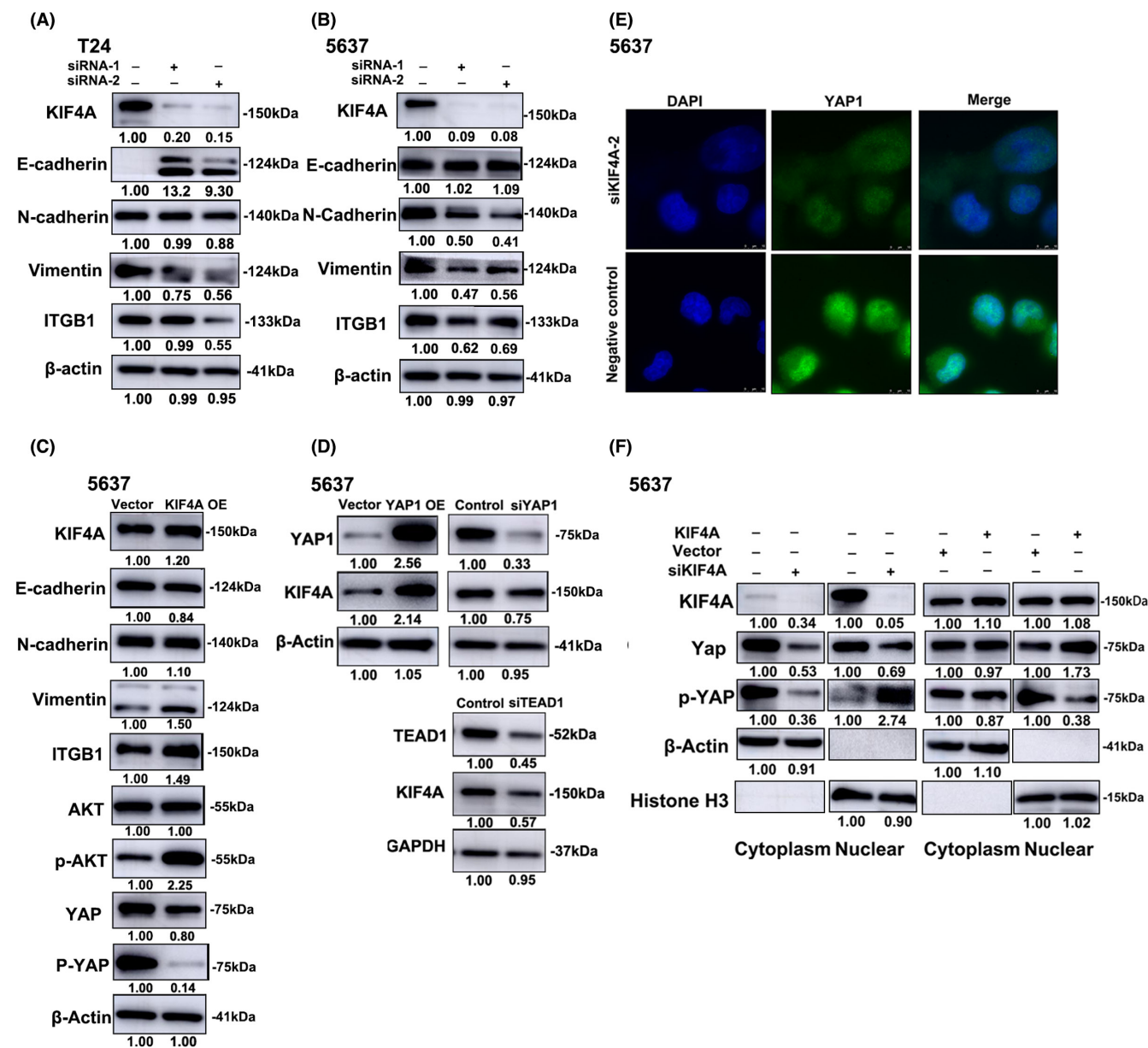




**FIGURE 2** Knockdown of KIF4A inhibited colony formation and proliferation while promoting apoptosis of UBC cells. (A,B) 5637 (A) and T24 (B) cells were transfected with siRNA-1, siRNA-2, or siRNA-3 against KIF4A. Western blot analysis was conducted to measure KIF4A protein level in the cells. (C–F) 5637 (C,E) and T24 (D,F) cells were transfected with siRNA-2 against KIF4A. Colony formation assay (C,D) and CCK-8 assay (E,F) were performed to examine cell proliferation. (G,H) 5637 (G) and T24 (H) cells were transfected with siRNA-1 or siRNA-2 against KIF4A. Flow cytometry analysis was carried out to examine cell apoptosis. (I,J) Western blot analysis was conducted to determine protein expression of p-AKT (Ser473), total AKT, Bcl-2, Bax, cleaved-PARP, PARP, cleaved-caspase-3, YAP1, p-YAP1, and P21 in 5637 (I) and T24 (J) cells.  $\beta$ -Actin was used as an internal reference. Data are expressed as the mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus negative control;  $n = 3$ .



**FIGURE 3** KIF4A promoted UBC cell migration and invasion. (A–D) T24 (A,B) and 5637 (C,D) cells were transfected with siRNA-1 or siRNA-2 against KIF4A. Transwell assay and wound healing assay were performed to examine cell invasion and migration. Representative images are shown. Magnification  $\times 100$ . (E) 5637 cells were transfected with lentiviral vectors expressing KIF4A. (F) Transwell assay and wound healing assay were performed to examine cell invasion and migration. (G,H) Quantification of F. (I,J) T24 (I) and 5637 (J) were transfected with siRNA-2 against KIF4A. A wound healing assay was performed to examine cell migration. Data are expressed as the mean  $\pm$  SEM. \* $p < 0.01$ , \*\*\* $p < 0.001$  versus negative control;  $n = 3$ . OE, overexpression.



**FIGURE 4** KIF4A regulated the protein level of EMT-related genes and interacted with Yes1 associated transcriptional regulator 1 (YAP1). (A,B) T24 (A) and 5637 (B) cells were transfected with siRNA-1 or siRNA-2 against KIF4A. Western blot analysis was performed to determine the protein level of KIF4A, E-cadherin, N-cadherin, vimentin, ITGB1, and β-actin. (C) 5637 cells were transfected with lentiviral vectors expressing KIF4A. Western blot analysis was conducted to determine the protein level of KIF4A, E-cadherin, N-cadherin, vimentin, ITGB1, AKT, p-AKT, YAP1, p-YAP1, and β-actin. (D) 5637 cells were transfected with lentiviral vectors expressing YAP1 or siRNA against YAP1 (siYAP1). Western blot analysis was conducted to determine the protein level of YAP1, KIF4A, TEAD1, β-actin, and GAPDH. (E) 5637 cells were transfected with siRNA against KIF4A. Immunofluorescence staining was performed to detect YAP1 protein level. (F) 5637 cells were transfected with lentiviral vectors expressing KIF4A or siRNA against KIF4A as indicated. Western blot analysis was carried out to determine the cytoplasmic and nuclear protein level of KIF4A, YAP1, and p-YAP1. β-actin and Histone H3 were used as cytoplasmic and nuclear internal references, respectively.

suppression of mitochondrial membrane translocation of Bax.<sup>23</sup> A recent study has shown that KIF4A silencing suppresses AKT activation in hepatocellular carcinoma.<sup>24</sup> Therefore, we hypothesized that the AKT pathway might be involved in KIF4A silencing-induced Bax upregulation in UBC cells. As expected, we found that KIF4A silencing

inhibited AKT phosphorylation in UBC cells, along with Bax upregulation. Thus, KIF4A possibly prevents cell apoptosis through AKT activation, which needs to be verified by AKT inhibition in future studies.

EMT is associated with increased ability of cell migration and invasion, playing a key role in cancer metastasis.<sup>25</sup>

Acquisition of EMT phenotypes is related to the activation of the PI3K/AKT/mTOR pathway.<sup>26</sup> However, the association of KIF4A with EMT in UBC remains unclear. In this study, overexpression or knockdown of KIF4A altered the protein level of EMT markers, suggesting that KIF4A could trigger EMT in UBC. In addition, KIF4A can transport ITGB1 in developing axons of cortical neurons.<sup>27</sup> Our results showed that knockdown of KIF4A suppressed protein level of ITGB1, whereas overexpression of KIF4A enhanced protein level of ITGB1 in UBC cells. ITGB1 can activate the AKT/FAK/YAP pathway during cell apoptosis and facilitate EMT.<sup>28,29</sup> PI3K/AKT has been shown to regulate tumor cell invasion and metastasis by promoting ITGB3 expression.<sup>30</sup> These findings, including ours, implies the involvement of ITGB1 in the role of KIF4A in apoptosis, migration, and invasion of UBC cells and further highlights the involvement of AKT and YAP1. However, these data need to be verified by loss of function analyses. In vivo studies are also required to assess the therapeutic potential of targeting KIF4A in UBC.

In conclusion, to the best of our knowledge, this study furtherly reported underlying mechanism that KIF4A facilitates cell proliferation, migration, and invasion of UBC cells and regulates the associated protein level of EMT, proliferation, and apoptosis-related proteins, such as p-AKT, ITGB1, p-YAP, and YAP1. These results suggest that KIF4A is a promising therapeutic target for UBC management.

#### AUTHOR CONTRIBUTIONS

**Chen Zhang:** Data curation (equal); formal analysis (equal); investigation (equal); resources (equal); writing – original draft (equal). **Maoyu Wang:** Data curation (equal); formal analysis (equal); investigation (equal); resources (equal); writing – original draft (equal). **Yidie Ying:** Data curation (equal); formal analysis (equal); investigation (equal); resources (equal); writing – original draft (equal). **Fang Meng:** Funding acquisition (equal); software (equal); validation (equal); visualization (equal). **Hongliang Gao:** Conceptualization (equal); software (equal); validation (equal). **Shuxiong Zeng:** Writing – review and editing (equal). **Yasheng Zhu:** Methodology (equal); writing – review and editing (equal). **Anwei Liu:** Writing – review and editing (equal). **Zhensheng Zhang:** Conceptualization (equal); methodology (equal); project administration (equal); supervision (equal). **Chuanliang Xu:** Conceptualization (equal); methodology (equal); supervision (equal).

#### FUNDING INFORMATION

This research was financed by National Natural Science Foundation of China (81772720, 81972391), and the “Voyaging Talents” Fund of The PLA Naval Medical

University (2021008149). The funder did not participate in the design of the study and collection, analysis, interpretation of data or in writing the manuscript.

#### CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

#### DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article.

#### ETHICS STATEMENT

The research was carried out in accordance with the Declaration of Helsinki. All clinical samples obtained were approved by the ethical board of Changhai Hospital (approval #: CHEC2019-134). Written informed consent was obtained from all patients.

#### ORCID

Chen Zhang  <https://orcid.org/0000-0001-6578-270X>

Zhensheng Zhang  <https://orcid.org/0000-0003-0299-3782>

#### REFERENCES

- Kaseb H, Aeddula NR. *Bladder Cancer*. StatPearls; 2022.
- Sylvester RJ, Rodríguez O, Hernández V, et al. European Association of Urology (EAU) prognostic factor risk groups for non-muscle-invasive bladder cancer (NMIBC) incorporating the WHO 2004/2016 and WHO 1973 classification systems for grade: an update from the EAU NMIBC guidelines panel. *Eur Urol*. 2021;79(4):480-488.
- Hollenbeck BK, Dunn RL, Ye Z, Hollingsworth JM, Lee CT, Birkmeyer JD. Racial differences in treatment and outcomes among patients with early stage bladder cancer. *Cancer*. 2010;116(1):50-56.
- Cao Q, Song Z, Ruan H, et al. Targeting the KIF4A/AR axis to reverse endocrine therapy resistance in castration-resistant prostate cancer. *Clin Cancer Res*. 2020;26(6):1516-1528.
- Taniwaki M, Takano A, Ishikawa N, et al. Activation of KIF4A as a prognostic biomarker and therapeutic target for lung cancer. *Clin Cancer Res*. 2007;13(22 Pt 1):6624-6631.
- Hu G, Yan Z, Zhang C, et al. FOXM1 promotes hepatocellular carcinoma progression by regulating KIF4A expression. *J Exp Clin Cancer Res*. 2019;38(1):188.
- Li T-F, Zeng H-J, Shan Z, et al. Overexpression of kinesin superfamily members as prognostic biomarkers of breast cancer. *Cancer Cell Int*. 2020;20:123.
- Zheng P, Wu K, Gao Z, et al. KIF4A promotes the development of bladder cancer by transcriptionally activating the expression of CDCA3. *Int J Mol Med*. 2021;47(6):99.
- Li Y, Zhu X, Yang M, et al. YAP/TEAD4-induced KIF4A contributes to the progression and worse prognosis of esophageal squamous cell carcinoma. *Mol Carcinog*. 2021;60(7):440-454.
- Mazumdar M, Sundareshan S, Misteli T. Human chromokinesin KIF4A functions in chromosome condensation and segregation. *J Cell Biol*. 2004;166(5):613-620.
- Li C, Hou X, Yuan S, et al. High expression of TREM2 promotes EMT via the PI3K/AKT pathway in gastric cancer:



- bioinformatics analysis and experimental verification. *J Cancer*. 2021;12(11):3277-3290.
12. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell*. 2007;129(7):1261-1274.
  13. Roseweir AK, Kong CY, Park JH, et al. A novel tumor-based epithelial-to-mesenchymal transition score that associates with prognosis and metastasis in patients with stage II/III colorectal cancer. *Int J Cancer*. 2019;144(1):150-159.
  14. Ribatti D, Tamma R, Annese T. Epithelial-mesenchymal transition in cancer: a historical overview. *Transl Oncol*. 2020;13(6):100773.
  15. Wang KJ, Wang C, Dai LH, et al. Targeting an autocrine regulatory loop in cancer stem-like cells impairs the progression and chemotherapy resistance of bladder cancer. *Clin Cancer Res*. 2019;25(3):1070-1086.
  16. Qi C, Min P, Wang Q, et al. MICAL2 contributes to gastric cancer cell proliferation by promoting YAP dephosphorylation and nuclear translocation. *Oxid Med Cell Longev*. 2021;2021:9955717.
  17. Jan R, Chaudhry GE. Understanding apoptosis and apoptotic pathways targeted cancer therapeutics. *Adv Pharm Bull*. 2019;9(2):205-218.
  18. Leibowitz B, Yu J. Mitochondrial signaling in cell death via the Bcl-2 family. *Cancer Biol Ther*. 2010;9(6):417-422.
  19. Zhang D, Lu W. KIF4A knockdown inhibits tumor progression and promotes chemo-sensitivity via induction of P21 in lung cancer cells. *Chem Biol Drug Des*. 2022. doi:10.1111/cbdd.14153
  20. Jin W, Ye L. KIF4A knockdown suppresses ovarian cancer cell proliferation and induces apoptosis by downregulating BUB1 expression. *Mol Med Rep*. 2021;24(1):516.
  21. Sun X, Chen P, Chen X, et al. KIF4A enhanced cell proliferation and migration via hippo signaling and predicted a poor prognosis in esophageal squamous cell carcinoma. *Thorac Cancer*. 2021;12(4):512-524.
  22. Lin L, Sabnis AJ, Chan E, et al. The hippo effector YAP promotes resistance to RAF- and MEK-targeted cancer therapies. *Nat Genet*. 2015;47(3):250-256.
  23. Stiles BL. PI-3-K and AKT: onto the mitochondria. *Adv Drug Deliv Rev*. 2009;61(14):1276-1282.
  24. Huang Y, Wang H, Lian Y, et al. Upregulation of kinesin family member 4A enhanced cell proliferation via activation of Akt signaling and predicted a poor prognosis in hepatocellular carcinoma. *Cell Death Dis*. 2018;9(2):141.
  25. Mittal V. Epithelial mesenchymal transition in tumor metastasis. *Annu Rev Pathol*. 2018;13:395-412.
  26. Chang L, Graham PH, Hao J, et al. Acquisition of epithelial-mesenchymal transition and cancer stem cell phenotypes is associated with activation of the PI3K/Akt/mTOR pathway in prostate cancer radioresistance. *Cell Death Dis*. 2013;4:e875.
  27. Heintz TG, Heller JP, Zhao R, Caceres A, Eva R, Fawcett JW. Kinesin KIF4A transports integrin  $\beta 1$  in developing axons of cortical neurons. *Mol Cell Neurosci*. 2014;63:60-71.
  28. Uchihara T, Miyake K, Yonemura A, et al. Extracellular vesicles from cancer-associated fibroblasts containing annexin A6 induces FAK-YAP activation by stabilizing  $\beta 1$  integrin enhancing drug resistance. *Cancer Res*. 2020;80(16):3222-3235.
  29. Li Y, Sun C, Tan Y, Zhang H, Li Y, Zou H. ITGB1 enhances the radioresistance of human non-small cell lung cancer cells by modulating the DNA damage response and YAP1-induced epithelial-mesenchymal transition. *Int J Biol Sci*. 2021;17(2):635-650.
  30. Shao N, Lu Z, Zhang Y, et al. Interleukin-8 upregulates integrin  $\beta 3$  expression and promotes estrogen receptor-negative breast cancer cell invasion by activating the PI3K/Akt/NF- $\kappa$ B pathway. *Cancer Lett*. 2015;364(2):165-172.

## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Zhang C, Wang M, Ying Y, et al. Knockdown of kinesin family member 4A inhibits cell proliferation, migration, and invasion while promoting apoptosis of urothelial bladder carcinoma cells. *Cancer Med*. 2023;12:12581-12592. doi:10.1002/cam4.5932